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Effects of Two Novel Peptides from Skin of *Lithobates Catesbeianus* on Tumor Cell Morphology and Proliferation

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1. Introduction

The number of resistant bacterial strains has continued to increase due to the overuse of antibiotics, necessitating continued development of novel antimicrobial agents. These resistant strains have made the care of infected patients with cancer more difficult [1, 2]. Indeed, infection control in patients with malignant tumors is one of the most important aspects of hospital management. Amphibian skin secretions contain a large number of antimicrobial peptides, including aurein 1.2, citropin 1.1, gaegurins, magainin, and magainin analogues, which have shown selective toxicity against human cancer cells [3, 4, 5]. There are now 105 antitumor peptides listed in the antimicrobial peptide database (APD, http://aps.unmc.edu/AP), 11 of which were isolated by Roaek [6] from the Australian bull frogs. Most of the remaining peptides have been isolated from the family magainins [7] or from Xenopus laevis.

Antimicrobial peptides rely on amphiphilic α-helix structures to associate with prokaryotic lipid bilayers, often forming transmembrane ion channels that cause cell leakage, mitochondrial failure, or the activation of apoptotic signaling cascades. Antimicrobial peptides have little effect on healthy eukaryotic cells, however, and tend to act selectively on prokaryotic cells and damaged eukaryotic cells. Thus, they have the capacity to kill bacteria, fungi, and viruses. In addition, antimicrobial peptides can damage cancer cells without damaging normal cells, possibly due to differences in the composition of the cell membrane and cytoskeleton. Whether the antimicrobial peptides kill bacteria and tumor cells through the same molecular mechanisms is uncertain [8]. In recent years, antimicrobial peptides have becoming an intense focus of research in zoology, physiology, and pharmacology [9, 10], as these peptide are high efficiency, low toxicity, broad-spectrum antimicrobial agents that may complement traditional antibiotics.

We isolated two novel polypeptides named temporin-La [11] and palustrin-Ca with strong antimicrobial and antitumor activities by screening the skin cDNA library of *Lithobates*

catesbeianus. In order to explore the antitumor mechanism of temporin-La and palustrin-Ca, we investigated the influence of these two polypeptides on HeLa ultrastructure by transmission electron microscopy. These two antimicrobial peptides are potential candidates for clinical trials as antibiotics and anticancer drugs.

2. Materials and methods

2.1 Materials

A *Lithobates catesbeianus* skin cDNA library was constructed in the Department of Microorganisms and Immunity, College of Animal Science and Veterinary Medicine, Jilin University. Tumor cells used in the anticancer assays were the gastric tumor cell lines SGC7901 and MGC-803, and the liver tumor cell lines SMMC7721 and BEL-7402. The tumor cells were obtained from the First Hospital of Jilin University. The Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Klebsislla pneumoniae* (ATCC700603), *Salmonella* (ATCC20020), and *Pseudomonas aeruginosa* (ATCC227853), the Gram-positive bacterium *Staphylococcus aureus* (ATCC25923), *Streptococcus suis 2* (CVCC606), *Listeria* (ATCC 54004), and *Bacillus subtilis* (ADB403), and the fungus *Candida albicans* (ATCC10231) were all obtained from China Institute of Veterinary drug Control.

2.2 Peptide synthesis

Temporin-La and palustrin-Ca were synthesized by GL Biochem (Shanghai, China) Ltd. according to the deduced amino acid sequences of mature peptides. High performance liquid chromatography and ESI-MS mass spectrometry confirmed that the purity of the synthetic peptides was higher than 95%. The synthetic peptides were dissolved in sterile water and used to evaluate antimicrobial and antitumor activities.

2.3 Antimicrobial assays

Microorganisms were incubated in LB broth at 37 °C to log-phase and then diluted in fresh LB broth to approximately 2×10^6 CFU/mL. To test the dose-response of the peptides, 50 μL samples of diluted microorganisms were mixed with 1:1 serial dilutions of the synthetic peptides in fresh LB in 96-well microtiter plates. Plates were incubated at 37 °C in a moist atmosphere for 16-18 h. After incubation, the absorbance of the plates at 600 nm was measured and recorded. The minimal inhibitory concentration (MIC) of the peptides was defined as the minimal concentrations at which no visible growth of the microorganisms was detected [12].

2.4 Hemolytic assays

The hemolytic effect of the toxins was tested using rabbit erythrocytes in liquid medium according to a method previously described [13]. Briefly, serially diluted peptides were incubated with washed rabbit erythrocytes at 37 °C for 30 min. Following incubation, the cells were centrifuged and the 595 nm absorbance of the supernatant was measured. Absorbance was compared to that of supernatant from cells completely lysed with 1% Triton X-100.

2.5 Transmission electron microscopy

Transmission electron microscopy was performed to study the potential antitumor mechanisms of temporin-La and palustrin-Ca on HeLa cells. Briefly, the tumor cells were

digested by trypsin and incubated in 50 mL cell culture flasks with toxin for 2 h. Cells were then harvested and centrifuged. The cell pellets were embedded, stained, dehydrated, and embedded in white resin. The embedded cells were then sliced, stained, and imaged using a JEM—1200EXll microscope under standard operating conditions.

3. Results

3.1 cDNA cloning of temporin-La and palustrin-Ca

Two bacterial clones encoding the two novel antimicrobial peptides were identified separately. The first clone encoded a mature peptide named temporin-La and the second encoded palustrin-Ca (Figure 1). The cDNAs were deposited in GenBank under accession numbers FJ430082 and FJ830669.

Temporin-La

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atgttccccttgaagaaatccctgttactccttttttttccttgggaccatcaacttatct   60
 M  F  P  L  K  K  S  L  L  L  L  F  F  L  G  T  I  N  L  S
Ttttgtgaggaagagagagatgtcgatcaagatgaaagaagagatgatccaggtgaaagg   120
 F  C  E  E  E  R  D  V  D  Q  D  E  R  R  D  D  P  G  E  R
Aatgttcaagtggaaaaacgattgttacgacatgttgtaaagattctcgaaaaatatttg   180
 N  V  Q  V  E  K  R  L  L  R  H  V  V  K  I  L  E  K  Y  L
Ggaaaataaccagaaatgttgaaactttgaaaatggaattggaaatcatttgatgtggaa   240
 G  K  *
Tattatttggctaaatgctcaacagatgttttataaaaataaataaatatgttgcaaaaa   300
Aaaaaaaaaaaaaaaaaaaaaaa                                       324
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Palustrin-Ca

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atgttcaccatgaagaaatccctgttgctccttttctttcttgggaccatctccttatct   60
 M  F  T  M  K  K  S  L  L  L  L  F  F  L  G  T  I  S  L  S
Ctctgtgagcaagagagagatgccgatggagatgaaggggaagttgaagaagtaaaaaga   120
 L  C  E  Q  E  R  D  A  D  G  D  E  G  E  V  E  E  V  K  R
Ggtttcctggatatcatcaaggatacggggaaggaatttgctgtgaaaattttgaataat   180
 G  F  L  D  I  I  K  D  T  G  K  E  F  A  V  K  I  L  N  N
Ttaaaatgtaaattggctggaggatgtccaccctgaatcagaagtcatctcatgtggaat   240
 L  K  C  K  L  A  G  G  C  P  P  *
Atcacttagctaaatctgtaatgtcttattaaaaaataaaaatatcacatgcaaaaaaaa   300
Aaaaaaaaaaaaaaaaaaaaaa                                        322
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Fig. 1. Nucleotide and deduced amino acid sequences of temporin-La and palustrin-Ca precursors. Sequences of the mature peptides are underlined. The asterisks represent the termination codon.

The deduced amino acid sequences, net charges, molecular masses, and isoelectric points of temporin-La and palustrin-Ca are shown in Table 1. Temporin-La consists of 13 amino acid residues with a net charge of +3 at pH 7.0. The C-terminus of temporin-La is amidated. A NCBI-BLAST search revealed that the nucleotide sequences of the precursors of temporin-La exhibit 40% sequence identity with other temporin precursors from *Rana* species. Palustrin-Ca consists of 31 amino acid residues with a net charge of +2 at pH 7.0. The seven peptide "Rana box" was formed by the Cys^{23} and Cys^{29}. Palustrin-Ca demonstrated approximately 50% homology with brevinin-2TD from the European frog and with ranatuerin-2Ca, ranatuerin-3, and ranatuerin 2Cb from bullfrog.

		Net charge (pH7.0)	Mr (Da)	pI
temporin-La	LLRHVVKILEKYLamide	+3	1623.08	9.70
palustrin-Ca	GFLDIIKDTGKEFAVKILNN LKCKLAGGCPP	+2	3303.97	8.79

Table 1. Primary structure, net charge, molecular masses, and isoelectric points of temporin-La and palustrin-Ca. The isoelectric point values were calculated using the ExPASy MW/pI tool (http://www.expasy.ch/tools/pi_tool.html). The molecular masses were determined by ESI-MS mass spectrometry.

3.2 Antimicrobial and hemolytic activities of temporin-La and palustrin-Ca

Minimum inhibitory concentrations (MICs) were determined to characterize antimicrobial activity (Table 2). Temporin-La and palustrin-Ca were both more effective against Gram-positive than Gram-negative bacteria. Temporin-La exhibited stronger inhibition against *S.aureus* and *S.suis* than palustrin-Ca. The MIC of temporin-La against *Staphylococcus aureus* was 7.8 μg/mL and 15.6 μg/mL against *Streptococcus suis*, while MICs for palustrin-Ca against these microbes were 7.8 μg/mL and 31.25 μg/mL. The antifungal activity [14, 15] of temporin-La, as assed by the MIC was 31.25 μg/mL. In contrast, palustrin-Ca was not nearly as potent as an antifungal agent with an MIC > 100 μg/mL. Temporin-La showed no measurable hemolytic activity against rabbit erythrocytes at 250 μg/ml. Similarly, palustrin-Ca showed little hemolytic activity (0.25% at 250 μg/ml).

Bacterium	Antimicrobial activity (MIC μg/ml) temporin-La	palustrin-Ca
Gram-positive bacteria		
Listeria ATCC 54004	ND	30
S. aureus ATCC 25923	2.5	7.8
S. suis 2 CVCC 606	15.6	31.25
Bacillus subtilis ADB403	>100	30
Candida albicans ATCC10231	31.25	>100
Gram-negative bacteria		
Salmonella ATCC 20020	15.6	>100
E. coli ATCC 25922	>100	ND
K. pneumoniae ATCC 700603	>100	60
P. aeruginosa ATCC 227853	60	30

Table 2. Antimicrobial activity of temporin-La and palustrin-Ca. The data represent mean values of three independent experiments. ND: not determined.

3.3 Temporin-La and palustrin-Ca possess strong anticancer activity

The antitumor activity of temporin-La and palustrin-Ca was evaluated by MTT assay (Table 3). The IC_{50} of temporin-La against SMMC7721 cells was 1.384 μg/mL, and palustrin-Ca was also a potent antitumor agent, with an IC_{50} of 0.951 μg/mL against SGC7901 cells.

Tumor cell	**Antitumor activity** IC_{50} (**μg/mL**)	
	temporin-La	palustrin-Ca
SGC7901	2.755	0.951
MGC803	3.937	1.572
SMMC7721	1.384	1.077
BEL7402	2.670	1.375
HeLa	4.685	1.202

Table 3. Antitumor activity of temporin-La and palustrin-Ca.

3.4 Electron microscopy

Transmission electron microscopy was performed to evaluate the possible cytotoxic mechanisms of temporin-La and palustrin-Ca (Fig. 2). After 24 h treatment with temporin-La or palustrin-Ca at 10×MIC, HeLa cells exhibited significant damage, with breached membrane integrity, cytoplasmic leakage, mitochondria vacuolization, cristae loss, and blurred boundaries.

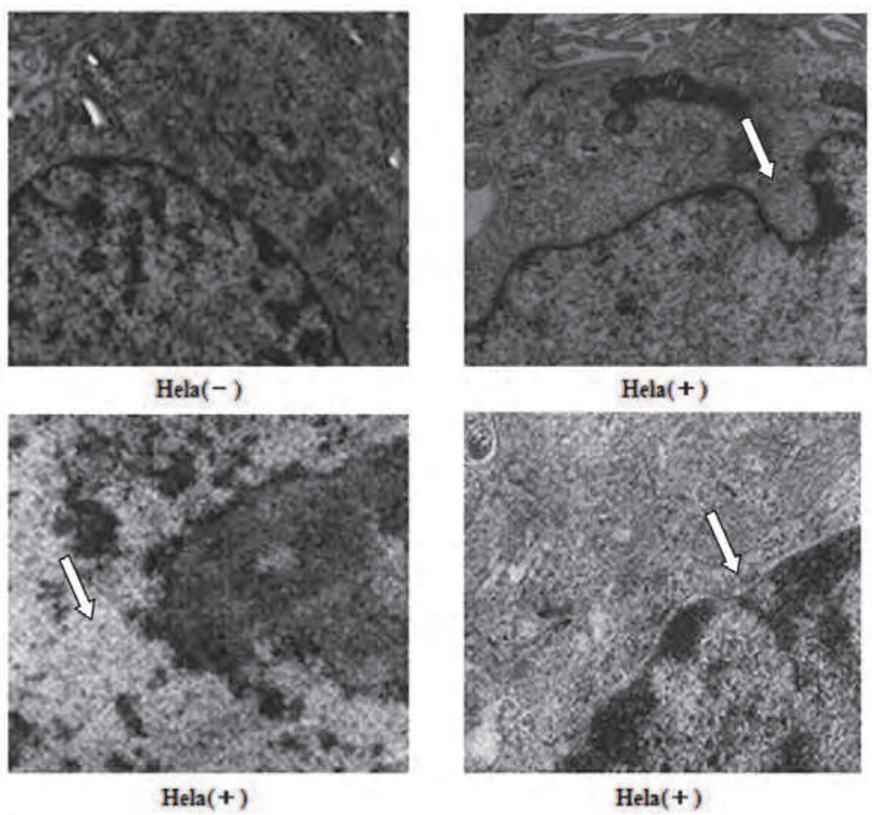

Fig. 2. The cytotoxic activity of temporin-La and palustrin-Ca against Hela cell *in vitro* (×20 000) Hela(-): untreated; Hela(+): temporin-La and palustrin-Ca treated. Peptides were used at 10 times the MIC. Arrows indicate the morphological changes.

4. Discussion

Antimicrobial peptides demonstrate selective toxicity against tumor cells [16, 17], but the cytotoxic mechanisms remain poorly understood. Recent studies suggest that the cytotoxic mechanisms of antimicrobial peptides against bacteria are distinct for these antitumor activities. The sensitivity of tumor cells to antimicrobial peptides is far higher than the normal cells, thus allowing selective antitumor effects. This selective cytotoxicity may be due to the higher acidic phospholipid content of tumor cell membranes and the reduced integrity of the cytoskeleton system. Tumor cells can be inhibited and killed by direct contact with antimicrobial peptides, suggesting that the effect on cancer cells may be due to insertion of antimicrobial peptides into the plasma membrane. Membrane insertion causes bilayer melting and membrane puncture, leading to leakage of intracellular contents, mitochondria vacuolization, cristae loss, blurred boundaries, chromosomal DNA breakage, inhibition of chromosomal DNA synthesis, and other detrimental events associated with membrane damage. Another possible mechanism of antimicrobial peptides is the induction of apoptosis. Mai *et al* [18, 19] showed that local injection of the peptide DP1 into mice quickly induced tumor cell apoptosis. On the other hand, the protective immune response can be enhanced by antimicrobial peptides, as shown by the resistance to the invaded cancer cells conferred by humoral immunity. Temporin-La and palustrin-Ca demonstrated significantly different antimicrobial and antitumor efficacies against the tested battery of bacteria, fungi, and tumor cell lines, suggesting that the cytotoxic mechanisms may be at least partially distinct for different targets.

Temporin-La had higher antibacterial activity against *Staphylococcus aureus, Streptococcus suis 2* and *Salmonella* than palustrin-Ca, with an MIC for *Staphylococcus aureus* of 2.5 μg/mL. The IC_{50} against the gastric cancer cell line SMMC7721 was only 1.384 μg/mL, and the IC_{50} values measured for all other tumor cells tested were lower than 5 μg/mL. This is far below the concentration required for mammalian red blood cell hemolysis, indicating that temporin-La can inhibit the growth of some Gram-positive bacteria and cancer cells while not damaging the membranes of other healthy mammalian cells. Palustrin-Ca had stronger antibacterial activity against *Staphylococcus aureus*, *Listeria,* and *Pseudomonas aeruginosa* than temporin-La. The MIC against *Staphylococcus aureus* was 7.8 μg/mL, and palustrin-Ca also exhibited promising inhibition against all the tumor cells studied, with IC_{50} values lower than 2 μg/mL. The IC_{50} against the liver cancer cell line SGC7901 was only 0.951 μg/mL, underscore the potential antitumor efficacy of palustrin-Ca. Temporin-La and palustrin-Ca are potential candidate antimicrobial and anticancer compounds that warrant further study.

5. Acknowledgements

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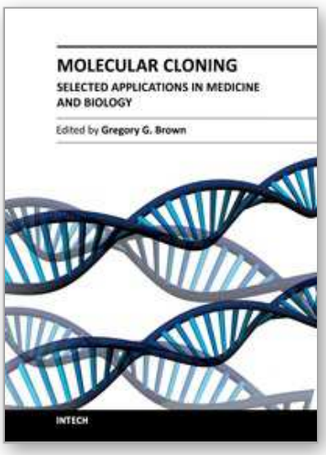

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The development of molecular cloning technology in the early 1970s created a revolution in the biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with a perspective on how pervasive the applications of molecular cloning have become. The contributions are organized in sections based on application, and range from cancer biology and immunology to plant and evolutionary biology. The chapters also cover a wide range of technical approaches, such as positional cloning and cutting edge tools for recombinant protein expression. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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